Pflugers Arch – Eur J Physiol (2005) 450: 53–60
DOI 10.1007/s00424-004-1374-2

EPITHELIAL TRANSPORT

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Effect of the mycotoxin, ochratoxin A, on hormone-stimulated ion transport in a cultured cell model of the renal principal cell

Received: 24 August 2004 / Accepted: 19 November 2004 / Published online: 4 January 2005

Abstract The mycotoxin ochratoxin A (OTA) is a common contaminant of many foodstuffs and, consequently, is present in a large proportion of tested populations of humans and commercial animals. The predominant effects of OTA are manifested in the kidney where the severity varies from salt wasting to renal carcinoma formation in a concentration-dependent fashion. The MDCK-C7 renal cell culture model responds to various hormones known to regulate electrolyte and fluid balance and was used as a model to study the chronic effects of an acute exposure to low dose OTA. The natriferic hormones aldosterone and insulin-like growth factor 1 (IGF1) both stimulate Na^+ flux in a reabsorptive direction via activation of the epithelial Na^+ channel (ENaC). In contrast, anti-diuretic hormone (ADH) stimulates three separate and temporally distinct ion transport responses, one of which is Na^+ reabsorption. Treatment of MDCK-C7 cells with OTA (100 nM) for 48 h selectively and irreversibly inhibits hormone-stimulated Na^+ reabsorption via ENaC. This effect was retained for 48 cell passages after the removal of the toxin and mimics the OTA-induced salt-wasting that has been documented in clinical studies. These studies indicate that the effect of the toxin is genomic and therefore, likely to be long lasting in exposed animals and humans.

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Keywords Aldosterone · Anti-diuretic hormone · Electrolyte homeostasis · ENaC · Epithelial sodium channel · IGF1 · Insulin-like growth factor 1 · MDCK-C7

Introduction

Ochratoxin A (OTA) is a fungal metabolite produced by several *Aspergillus* and *Penicillium* species. Because of the ubiquitous presence of these fungi, the toxin is commonly found in grains, coffee, wine, animal feed and animal products [3, 4, 5]. OTA is remarkably stable and remains effective after processing treatments such as cooking, brewing or roasting. In addition, binding of the toxin to serum proteins such as albumin ensures a relatively long half life in the blood [4]. In populations such as Europe, Canada, the United States and northern Africa, where animal and human serum levels have been monitored, there is a high incidence of measurable to toxic levels in the population [4, 11, 12, 16, 20].

The toxin appears to concentrate in certain organs, notably the kidney. Concomitantly, the kidney is also one of the major organs affected by OTA. In vivo studies in rats have demonstrated a very sensitive effect of OTA on post-proximal nephron function [5] with subsequent studies indicating that acute exposure has a predominant effect on reabsorptive transport functions in the distal nephron [4]. The nature of the effect on electrolyte and nutrient excretions, predominately a toxin-induced increase in NaCl excretion and a decrease in K^+ excretion, confirm an action on the distal nephron/ collecting duct.

Chronic or high level exposure has more global renal effects including reduction in renal blood flow and glomerular filtration, proximal and distal tubular function, and the development of carcinogenic properties [4, 5]. In areas of very high levels of human exposure, epidemiological studies have linked OTA as the causative agent in Balkan endemic nephropathy, renal adenomas and carcinomas [12, 16, 21]. The epidemiological studies have

been supported by animal and tissue culture studies demonstrating specific uptake by renal cells [18], effects of OTA on growth and apoptosis in renal epithelial cells [7, 9, 10], effect of hormonal signaling via epidermal growth factors [1] or extracellular signal-regulated kinases, ERK [18] and induction of oxidative stress and proximal tubular toxicity [17].

Many of the in vitro studies use levels of the toxin that exceed typical concentrations of in vivo exposure. It is of interest, therefore, to explore the long-term effects of physiologically relevant toxin concentrations. Previous studies have shown that acute exposure to nanomolar concentrations of OTA altered the ability of the cells to regulate pH by inhibiting anion conductance. Dome formation in MDCK (Madin Darby canine kidney) cells was also inhibited indicating an effect on salt and water movement [4, 5, 8]. When a principal cell subclone of the MDCK line (MDCK-C7) was exposed to 100 nM OTA for 48 h, the resultant cell line showed irreversible changes in both function and genotype. The predominant changes in function were a diminished basal K^+ secretory rate and a loss of responsiveness to aldosterone. In addition, the OTA-treated cells maintained a higher transepithelial resistance than the naïve MDCK-C7 line. The genotypical changes appeared to be numerical aberrations with no specific changes in karyotype [8].

In the present studies we have extended the experiments using the OTA-treated MDCK-C7 cells. After the single exposure, the functional effects on the cell line were monitored for an additional 48 passages over the course of several years. We found a specific and irreversible defect in reabsorptive Na^+ transport in response to hormonal stimuli. The permanency of the change suggests an alteration which is likely mediated at the genomic level. Cellular viability as well as other ion transport responses remained unchanged.

Materials and methods

MDCK-C7 cell culture:

The MDCK-C7 cell line is a subclone of the heterogeneous MDCK cell line. This subclone has the characteristics of the principal cells of the distal nephron [6] and responds to hormonal stimuli known to regulate ion transport in the distal nephron [2,13]. The MDCK-C7 cells form a high resistance ($>1{,}000\ \Omega/cm^2$) monolayer when grown on permeable supports.

The MDCK-C7 cells were maintained in a 37°C incubator with 5% CO_2 and 95% O_2 gas mixture. Cells were carried in 75-ml flasks and fed with MEM with Earle's salt, nonessential amino acids and L-glutamine (MEM; Gibco/BRL, Grand Island, N.Y., USA) supplemented with 10% fetal bovine serum (Sigma, St Louis, Mo., USA), 26 mM $NaHCO_3$, 25 U/ml penicillin and 25 μg/ml streptomycin and adjusted to pH 7.0. Cellular media was changed thrice weekly and the cells were subcultured by trypsinization every 5–7 days. For functional assays, the cells were seeded ($\sim 5.5\times10^4$ cells/cm^2) onto Nucleopore polycarbonate membranes forming the bottom of Transwell chambers (Costar, Cambridge, Mass., USA). The Transwell chambers form a two-compartment system in which media can be added to both apical and basolateral surfaces. The cells were used 7–11 days after plating onto the permeable supports.

To study the effect of OTA, the cells were treated for 48 h with 100 nM OTA and subsequently cultured in OTA-free media [8]. The cells were grown on culture flasks or permeable supports, trypsinized, frozen in liquid N_2, re-extracted from the liquid N_2 and plated onto permeable supports for 48 passages after the initial treatment with OTA.

Functional assays

Standard Ussing-style electrophysiological techniques were used to examine net ion transport across the cellular monolayers [2, 13, 23]. Nucleopore filters containing confluent monolayers were removed from the Transwell chambers and clamped between the halves of an Ussing chamber (World Precision Instruments, Sarasota, Fla., USA). The chamber contains ports for insertion of voltage electrodes (close to the tissue) and current electrodes (at opposite ends of the chamber). The chamber also contains ports for attachment to water-jacketed buffer reservoirs where the buffer is maintained at 37°C and circulated through the electrophysiology chamber by means of a 5% CO_2/O_2 gas lift. Unless otherwise stated, the bathing media was serum-free MEM. For the ion substitution experiments, the control cultures were equilibrated and measured in Kreb's/Henseleit media (116.4 mM NaCl, 25 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 4.1 mM KCl, 0.6 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11.1 mM glucose and 1.2 mM HCl, pH 7.1). The Cl^- free cultures were incubated and measured in Kreb's/Henseleit in which Cl^- was replaced with NO_3^-. The electrodes were connected to a voltage-clamp amplifier (Current Voltage Clamp; World Precision Instruments) for measurement of net ion flux as short-circuit current (SCC). Transepithelial resistances were calculated every 200 s by applying a 2-mV pulse across the epithelium and measuring the resultant deflection in SCC. Cultures were used only if the resistance was $\geq 1{,}000\ \Omega/cm^2$. The calculated potential difference across the epithelia changes in parallel with the change in SCC.

Cultures were monitored in the Ussing chambers under short-circuit conditions for at least 0.5 h or until a stable baseline current was obtained. Hormones were added to the serosal bathing media; amiloride (10^{-5} M) was added to the apical bathing media. The data are presented as mean ± SEM with n indicating the number of experiments. Student's *t*-test was used for statistical comparison with $P \leq 0.05$ considered significant.

Analysis of ENaC subunit protein expression

MDCK-C7 and OTA-treated MDCK-C7 cells were grown to confluence on permeable supports (11–19 days), washed three times with cold serum-free media and scraped from the filter support in 500 µl of lysis buffer (0.14 mM SDS, 10% glycerol, 1 mM DTT, in 0.05 M TRIS pH 6.8, 5% protease inhibitor cocktail). To effect solubilization, cell lysates were sonicated for 20 1-s pulses with a titanium-tip sonicator (20% duty cycle, Granson Sonifier 450) and stored at −20°C.

Cell lysates were separated using 7.5% polyacrylamide gels and subsequently transferred to PVDF membranes. One of a duplicate pair of the PVDF membranes was stained with Coomassie brilliant blue protein stain to show equal sample loading. The other duplicate PVDF, used for antibody probing, was washed once in 1×TRIS-buffered saline (TBS) pH 7.5 for 10 min then blocked with 5% nonfat milk in TBS, pH 7.5 for 1 h. The PVDF was washed again in TBS for 10 min then incubated in the primary antibody solution (α, β, or γ ENaC [22]) in 0.5% milk in TBS overnight at 4°C. The PVDF was washed again in TBS for 10 min and then incubated with goat anti-rabbit antibody conjugated with horseradish peroxidase diluted in 0.5% milk in TBS for 1.5 h. A series of six to seven washes in TBS for 10 min occurred after the secondary antibody incubation, the second of which was in 0.05% Tween-TBS, pH 7.5. For viewing specific bands, SuperSignal West Dura Extended Duration Substrate (Pierce Chemical, Rockford, Ill., USA) was used according to the manufacturer's protocol and exposed on Hyperfilm (Amersham, Piscataway, N.J., USA) to view the antibody binding.

Results

MDCK-C7 cells were treated with 100 nM OTA in normal serum-free growth media for 48 h. The cells were then further cultured in the absence of OTA for an additional 48 passages. During the culturing procedure, cells were also placed in liquid N_2 for long-term storage, thawed and plated. The treated cells were indistinguishable from the untreated MDCK-C7 cells in growth and viability characteristics in response to all culturing conditions.

We have previously shown that the MDCK-C7 cell line demonstrates natriferic (Na^+ retaining) responses to aldosterone (10^{-6} M) and IGF1 (insulin-like growth factor 1; 100 nM) [2]. After treatment with OTA, the responses to these two hormones were completely abolished and the cells had no amiloride-sensitive current either before or after hormone stimulation. This specific loss of transepithelial Na^+ transport was maintained throughout all the subsequent passages. Figure 1 shows a comparison of the responses to IGF1 and aldosterone in age-matched naive and OTA-treated MDCK-C7 cells.

The MDCK-C7 cell line has a multiphasic response to ADH (anti-diuretic hormone). Three temporally separable transport events can be distinguished. In chronological order over a 30-min time frame these are, a transient anion secretion via CFTR (cystic fibrosis transmembrane regulator), a verapamil- and Ba^{2+}-sensitive anion secretion or cation absorption and Na^+ reabsorption via the epithelial Na^+ channel (ENaC) [13]. This response is depicted in Fig. 2, top panel. In Fig. 2 (bottom panel) the responses of age-matched MDCK-C7 cells which had been previously treated with OTA are illustrated. The first two electrogenic responses are very similar. Interestingly, however, the third ion transport response, that of Na^+ flux via ENaC, is missing in the OTA-treated cells as evidenced by the lack of amiloride-sensitive transport after 30 min. This lack of amiloride-sensitive transport in the basal as well as ADH-treated cells was also present if the amiloride was added to OTA treated cells 15 min after hormone addition (Fig. 3).

Previous studies suggested that the nature of the initial transport peak is Cl^- and/or bicarbonate secretion through the CFTR [13]. In contrast to Na^+ transport, the anion secretion is unchanged in magnitude or nature of the ion transported in OTA-treated cells. In accordance with previous studies, NPPB [5-nitro-2-(3-phenylpropylamino)-benzoic acid], an inhibitor of CFTR diminishes the first, transient peak in OTA treated cells (Fig. 4, top panel). Pre-incubation in Cl^--free media (bilaterally) also diminishes the size of the response (Fig. 4 , bottom panel).

The various components of the study shown in the figures above were conducted using cells from 16–48 passages after treatment with OTA. A summary of amiloride-sensitive transport is shown in Fig. 5.

A parsimonious explanation for the specific effect on ENaC-mediated Na^+ reabsorption would be that the toxin inhibited the synthesis of one or more of the three (α,β,γ) subunits comprising the amiloride-sensitive Na^+ channel. Using subunit-specific antibodies for each individual subunit [22], we demonstrate that the synthesis and expression of these proteins is not substantially altered in the OTA-treated cells (Fig. 6). For the α and β subunits there was sufficient material to detect both the core protein and the fully glycosylated forms of the subunit while the γ subunit was detected predominately as the fully glycosylated protein. In all cases, the subunit proteins in the OTA-treated cells were present in abundances that were equal to or greater than identical proteins found in the parental cell line.

The treatment with OTA had no adverse effect on the high-resistance nature of the MDCK-C7 cell line. In fact, the cells which were treated with the OTA had a significantly higher transepithelial resistance (Fig. 7).

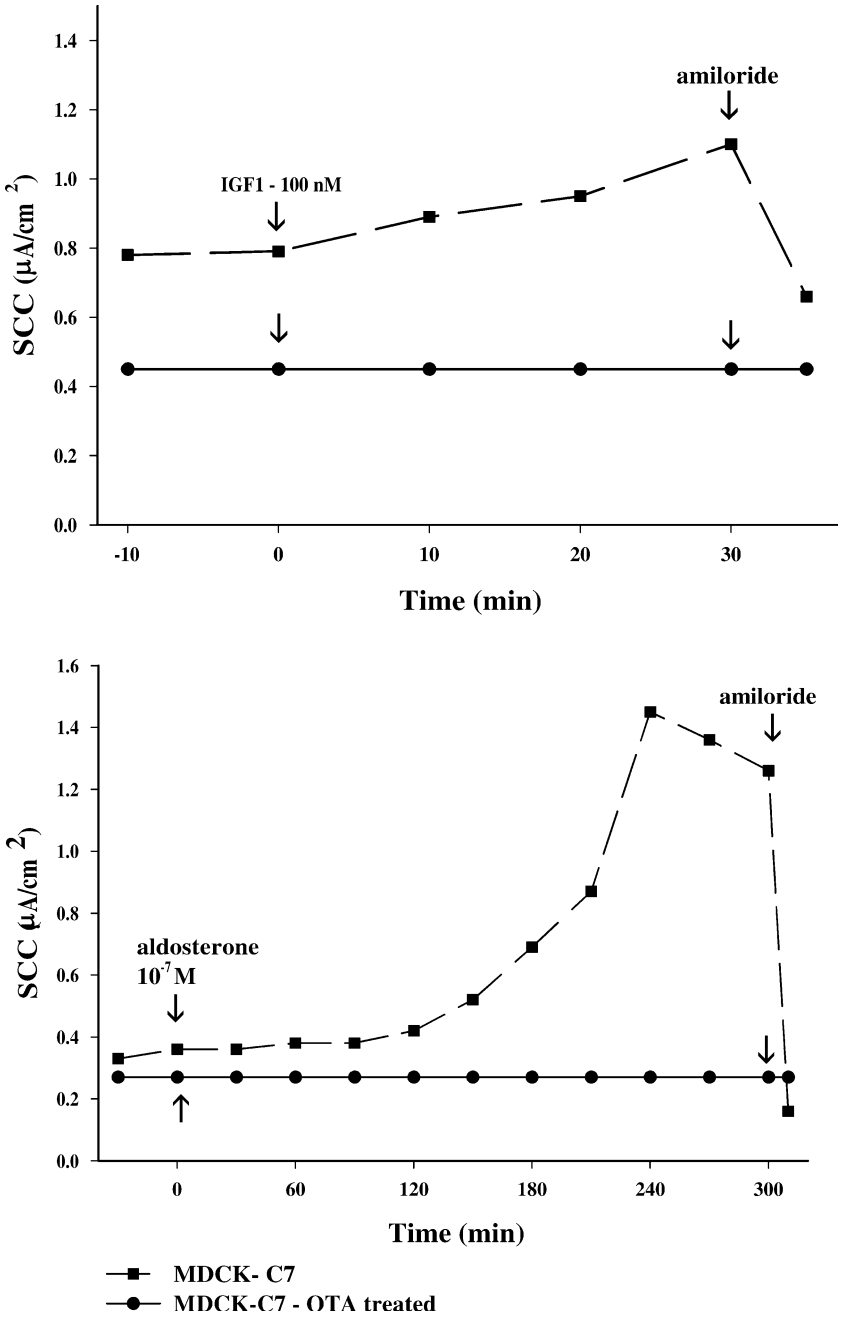

Fig. 1 Comparison of Na^+ transport responses to IGF1 and aldosterone in naïve and ochratoxin-A (OTA)-treated MDCK-C7 cells. In these representative experiments, ion transport was measured as short-circuit current (SCC) in age-matched (9–10 days) cultures grown on permeable supports and mounted in modified Ussing chambers. For the electrophysiological studies, the cells were bathed in serum-free media. The *top panel* illustrates the response to 100 nM IGF1 added to both cultures at time $t=0$. Thirty minutes later amiloride (10^{-5} M) was added to both cultures to determine the proportion of the SCC due to Na^+ transport via epithelial Na^+ channels (ENaC). The *bottom panel* illustrates the response to 10^{-7} M aldosterone added to both cultures at time $t=0$. Five hours later amiloride (10^{-5} M) was added to both cultures as indicated by the *arrows*

Discussion

Ingested OTA is concentrated in the kidney and has been implicated as a nephrotoxin. Previous studies have explored the effect of relatively high concentrations of OTA in order to determine the nature of the toxic actions of this compound. However, low level exposures are more applicable to what is normally found in the majority of the population. Based on previous studies, one of us (M.G.) has suggested that at physiologically relevant concentrations, OTA does not act as a classic toxin by altering cell viability but, rather, by modulating renal cell function [1, 8].

We have modeled the low-dose, subtoxic cellular effects of the mycotoxin by studying functional consequences in a principal cell line which responds to hormonal stimuli by expressing polarized ion transport. The dose chosen, 100 nM, was previously shown to be devoid of effects on growth inhibition, protein content, or transepithelial resistance in polarized, absorptive intestinal cells [14]. In the MDCK-C7 cell line, 100 nM OTA caused both phenotypic and genotypic changes as previously reported [8]. After OTA treatment and subsequent culture in OTA-free media, the cells demonstrated numerical chromosomal aberrations, reduction in electrolyte transport (K^+ secretion), a loss of

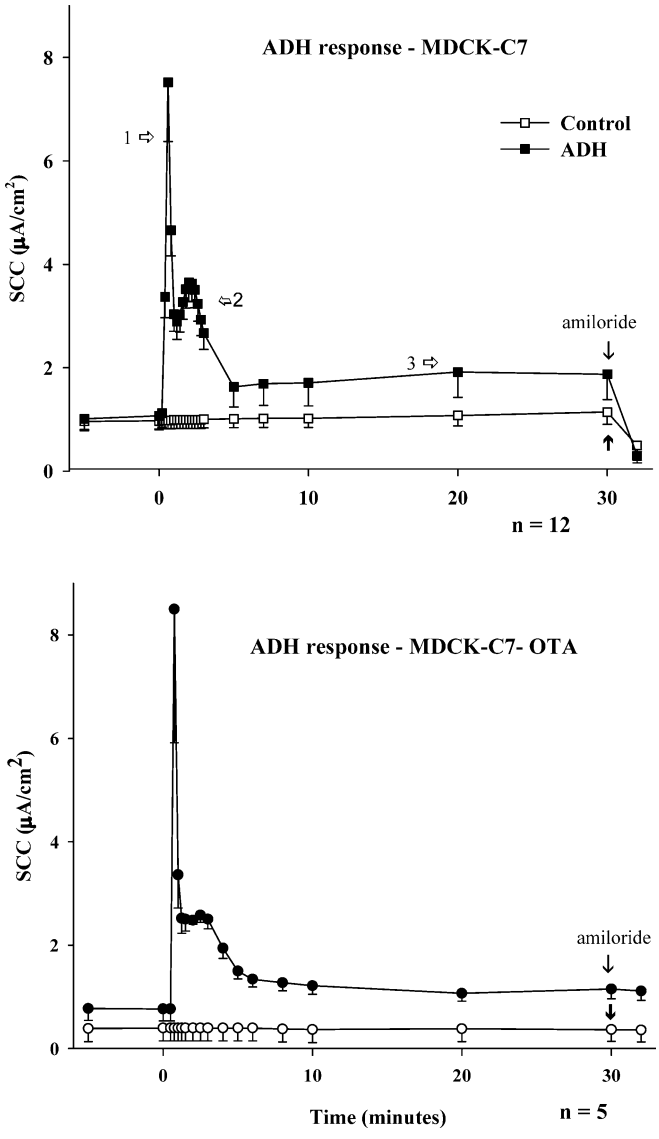

Fig. 2 Comparison of ion transport responses to anti-diuretic hormone (ADH) in naïve and OTA-treated MDCK-C7 cells. Ion transport was measured as SCC. For the electrophysiological studies, the cells were bathed in serum-free media. ADH (10 mU/ml) was added to the serosal bathing media as indicated at time $t = 0$. Amiloride (10^{-5} M) was added to the apical bathing media of all cultures at time $t = 30$ min. *Symbols* denote the mean ± SEM. The SEM bars are shown in the negative direction only. n = number of experiments. The three distinct transport phenomena are indicated by *open arrows* on the *top panel*. The third of these transport events, an amiloride-sensitive component, is absent in the experiments illustrated in the *lower panel*

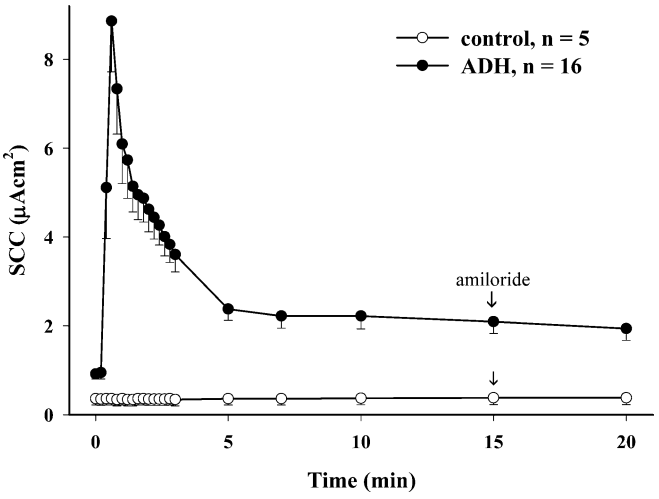

Fig. 3 Short-term analysis of ion transport responses to ADH in the MDCK-C7 cells which had been treated with OTA. Ion transport was measured as SCC. For the electrophysiological studies, the cells were bathed in serum-free media. ADH (10 mU/ml) was added to the serosal bathing media as indicated at time $t = 0$. Amiloride (10^{-5} M) was added to the apical bathing media of all cultures at time $t = 15$ min. *Symbols* denote the mean ± SEM. The SEM bars are shown in the negative direction only. n = number of experiments

responsiveness to aldosterone and an increase in transcellular resistance [8].

In response to aldosterone or IGF1, confluent MDCK-C7 cell monolayers exhibit an increase in transepithelial Na^+ transport in a reabsorptive direction [2]. Here we have shown that the natriferic responses to both the steroid or peptide hormones were completely and irreversibly inhibited by the single OTA exposure. Surprisingly, the hormonal responses did not return when the cells were continued in culture for 48 passages.

These results are indicative of a genomic alteration in the pathways controlling electrolyte transport in the cell line. Next, we examined whether this change was global, affecting multiple transporters, or whether it was more specific. To discern between these possibilities, we examined the acute response to ADH, a hormone that stimulates the activity of three different transport phenomena in a 30-min time frame in the MDCK-C7 cell line [13].

Interestingly, two of the three transport systems stimulated by ADH exhibited normal responses after OTA treatment. The third, an increase in amiloride-sensitive Na^+ flux, was inhibited in the OTA-treated cells. The initial transport phenomena, anion secretion via CFTR, was present in the toxin-treated cells and had the characteristics which we had previously demonstrated in untreated cells. Therefore, the genomic effect of low dose OTA seems to be a selective inhibition of transport initiated via the apically situated ENaC. Activation of ENaC secondarily stimulates K^+ secretion, thus accounting for the previous observations regarding the K^+ secretory response [4, 8].

Interestingly, the inhibition of transport is not due to a decreased expression of the ENaC subunit proteins. All subunits appear to be present in the OTA-MDCK-C7 cells in amounts similar to, if not higher than, those of the untreated line. Additional studies will be necessary to elucidate the mechanism of action of the toxin and such studies may also provide additional important information regarding the regulation of ENaC, a channel which is clinically important because of its role in the regulation of blood pressure.

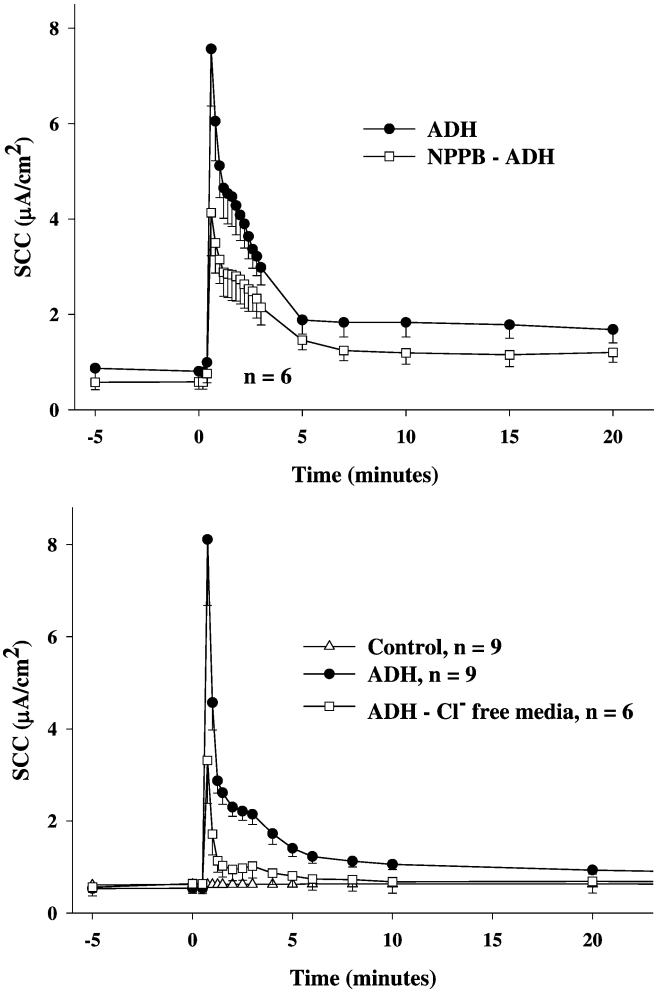

Fig. 4 Characterization of the initial transport response in MDCK-C7 cells which had been treated with OTA. For the electrophysiological experiments, cells were bathed in Kreb's Henseleit media. OTA-treated cells were preincubated with NPPB (500 μM) for 30 min (*top panel*) or incubated in Cl^--free media (bilaterally) for 1–2 h (*bottom panel*) before the addition of ADH (10 mU/ml) to the serosal bathing media at time $t = 0$. In the experiment depicted in the *top panel*, amiloride (10^{-5} M) was added to the apical bathing media of cultures at time $t = 15$ min. In the experimental set depicted in the *bottom panel*, amiloride was added at time $t = 30$ min. The amiloride-sensitive current was not shown in the bottom panel but is negligible. *Symbols* denote the mean ± SEM. The SEM bars are shown in the negative direction only. n = number of experiments

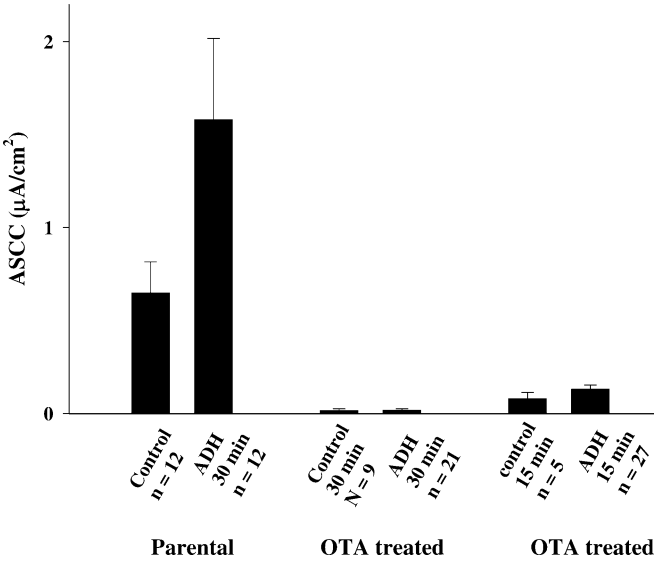

Fig. 5 Amiloride-sensitive SCC (*ASCC*) in MDCK-C7 cells as compared with OTA-treated MDCK-C7 cells. *Bars* represent the mean ASCC from a compilation of experiments using naïve MDCK-C7 cells or OTA-treated MDCK-C7 cells 16–48 passages after treatment with the toxin. *Bars* are shown as mean ± SEM

The functional effects which we have described in our electrophysiological studies correlate with the observed clinical effects of increased Na^+ excretion and decreased K^+ excretion [4]. Furthermore, our results suggest that a single exposure to OTA will have long-term effects on Na^+ transport in the principal cells since the mycotoxin appears to exert an irreversible effect on function in the exposed cells. This may have serious consequences for salt and water balance since these are the hormone-responsive cells in which transport is regulated in order to maintain fluid and electrolyte homeostasis.

OTA has been shown to have additional effects on other renal cell types. In an MDCK model of the intercalated cells, the acute effect of OTA appeared to be a blockage of anion conductance [5]. Moreover, in human immortalized kidney epithelial cells, nanomolar OTA potentiated hormone-induced Ca^{2+} signaling and proliferation [1].

OTA also selectively modulates transport in other polarized epithelial cells. In cultured human intestinal cell lines, HT-20–04 and Caco-2–14, micromolar concentrations of OTA inhibited Na^+-dependent glucose transport, stimulated the active transport of serine and had no effect on cAMP-dependent Cl^- transport through the CFTR [14]. The latter result agrees with our own findings of selective inhibition of Na^+ transport with no effect on anion secretion via CFTR. Thus, OTA may modulate various cellular transport systems and the effects may be cell-type specific. Additional studies will be required to determine whether the changes in other cell types are also irreversible.

In contrast to studies employing higher toxin concentrations, the functional effects that we observed were not accompanied by decreases in transepithelial resistance. In fact, the toxin-treated cells exhibited a statistically higher transepithelial resistance. This increased resistance was maintained throughout the 48 passages and is likely reflective of the reduced Na^+ and K^+ transport which are the major ion movements under basal (non-hormone treated) conditions.

In primary cultures of proximal tubule cells, nanomolar concentrations of OTA had no effect on cell viability or formation of transepithelial Na^+ or K^+ gradients. Conversely, micromolar concentrations reduced cell

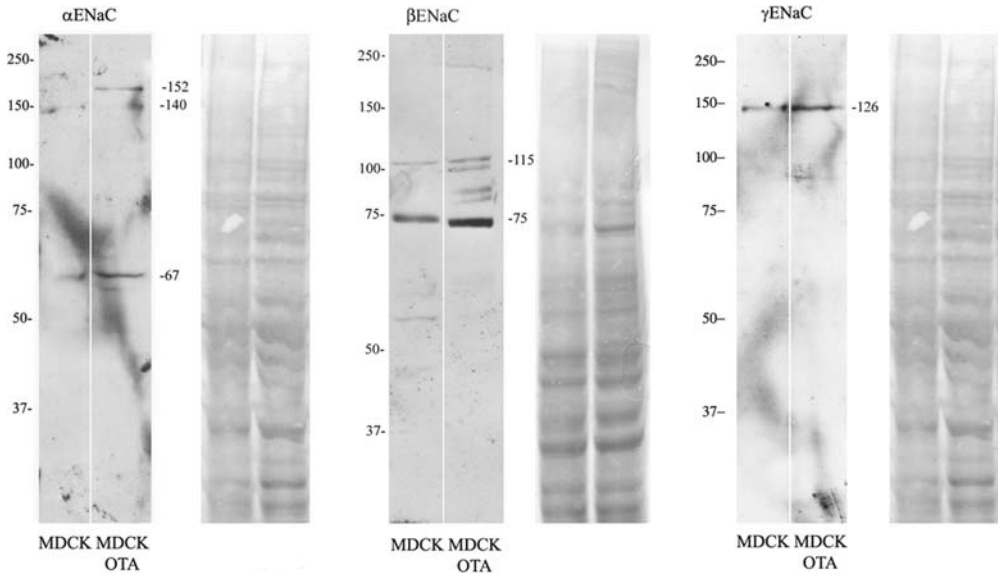

Fig. 6 Expression of ENaC subunits in MDCK-C7 and OTA-treated MDCK-C7 cells. Total cell lysates were prepared separated by polyacrylamide gel electrophoresis (PAGE). In each case duplicate gels of identical lysates were generated. One set was used for immunodetection using subunit specific antibodies as indicated (*left-hand pair*); the second set was stained with Commassie brilliant blue protein stain to verify equal protein loading (*right-hand pair*). The molecular weights (in kDa) shown on the *left* of the gel sets indicate the location of the molecular weight markers; the molecular weights shown on the *right* of the gel sets indicate the approximate size of the detected bands as calculated from the standard curve generated from the molecular weight markers

viability, monolayer integrity and transepithelial ion gradients [7]. These studies were substantiated in the LLC-PK1 proximal tubule cell line, where micromolar concentrations of OTA also caused a decrease in the barrier function of the epithelial monolayer [10]. In separate studies, micromolar concentrations also decreased the transcellular resistance of intestinal epithelial cells [14]. Thus, there appears to be a biphasic effect on resistance with nanomolar concentrations increasing, and micromolar concentrations decreasing, the magnitude of the transepithelial resistance.

The biphasic changes in transepithelial resistances may parallel the clinical changes that are noted after OTA exposure. Low dose exposure has been shown to have an effect on renal electrolyte transport and we have modeled one aspect of that process here. This would logically be expected to be paralleled by an increase in resistance in tight epithelial monolayers. On the other hand, decreases in transepithelial resistance indicate a loss of junctional integrity and, thus, an increase in junctional permeability. The loss of cellular resistance has interesting clinical implications that may explain some of the more toxic effects of high level exposure to OTA. A loss of the barrier function of epithelial cells has been postulated to contribute to the development of malignancies [15]. Growth factors are small molecular weight polypeptides which are easily filtered by the glomeruli. Peptide hormones and growth factors in the filtrate normally have no access to their cognate receptors on the basolateral side of renal tubular epithelial cells. However, as suggested by Mullin [15], when the barrier function of the epithelial monolayer is breached, the high concentration of growth factors which can interact with the basolateral receptors may contribute to the malignant transformation. This alteration of transepithelial permeability may be one of the factors responsible for the increased incidence of renal carcinoma observed in instances of chronic, high exposures to OTA.

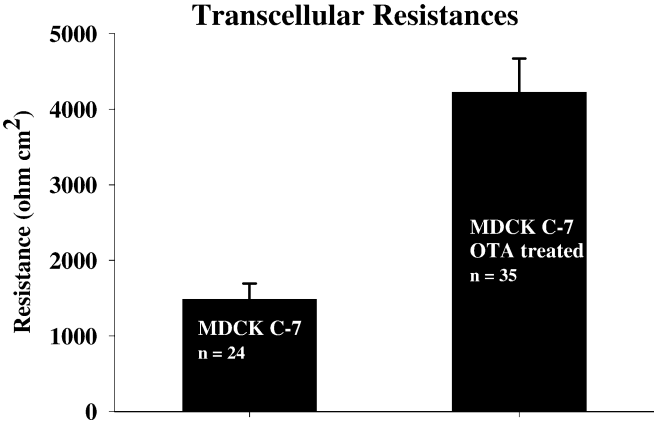

Fig. 7 Transcellular resistances of naïve MDCK-C7 cells or OTA-treated MDCK-C7 cells 16–48 passages after treatment with the toxin. The mean resistances of the OTA-treated cells are statistically higher than the parental line ($P < 0.000015$)

In summary, our studies correlate with the clinical observations of salt wasting after exposure to low doses of OTA. Furthermore, these results indicate that the effects of low dose toxin exposure appear to be irreversible and may, therefore, reflect genomic changes.

Acknowledgements The C7 subclone was a kind gift from Dr. H. Oberleithner, University of Muenster, Germany. The ENaC

antibodies were generously supplied by Dr. Caroline Ecelbarger, Georgetown University, Washington, D.C., USA. These studies were supported by a Veteran's Affairs Merit Review Grant (B.B.-Y.), UROP grants from IUPUI and a grant from the Deutsche Forschungsgemeinschaft (Ge 905/3-4; M.G.)

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